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STRUCTURE-FUNCTION RELATIONSHIP OF THE DEVELOPING ACETYLCHOLINE RECEPTOR IN AMPHIBIANS

A

THESIS

Presented to the Faculty

Of the University of Alaska Fairbanks

In Partial Fulfillment of the Requirements

For the Degree of

DOCTOR OF PHILOSOPHY

By

Michael Sullivan, B.A., M.D.

Fairbanks, Alaska

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By

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ABSTRACT

The nicotinic acetylcholine receptor is responsible for the transfer of signals from the peripheral nervous system to skeletal muscle, resulting in movement. Despite the importance of the acetylcholine receptor, many questions remain unanswered about the relationship between the structure and function of the receptor. The purpose of our research was to explain certain features of the relationship between the amino acid sequence and the function of the amphibian nicotinic acetylcholine receptor. We focused on describing the structural elements underlying the physiologic changes that occur during development and re-innervation of damaged mature muscle. We used molecular biological techniques to alter the amino acid sequence of the receptor and then studied the effects of these alterations using the electrophysiological technique of single channel recording. Our research resulted in the discovery of critical residues involved in two important characteristics of receptor function, the conductance and open time of the ion channel. These results offer new molecular insights into the classic observation that synaptic currents become briefer in duration during the course of muscle development in vertebrates.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

Acknowledgments

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Throughout this thesis I use the word "we" to describe the experimental work. I do this to acknowledge Rick Kullberg and Jesse Owens. Rick and Jesse have made remarkable contributions in the field of developmental neurobiology. Their previous work and experience served as the basis for all of my research. While the physical labor was primarily my own, the ideas, planning and interpretation were group efforts. Jesse taught me the technique of single channel recording and served as an invaluable resource for consultation and direction throughout this work. Rick was my primary advisor who taught me not just about the amphibian acetylcholine receptor, but also about the principles and ethics of research. He also provided mentorship, friendship and inspiration, all of which were needed for the successful completion of my studies.

CHAPTER 1
INTRODUCTION

The nicotinic acetylcholine receptor synapse is one of the best-studied chemical synapses in the nervous system. The nicotinic acetylcholine receptor is responsible for the transfer of signals from the motor neuron system to skeletal muscle resulting in movement. The receptor is also an abundant receptor in the brain and spinal cord. It has been implicated in many diseases including myasthenia gravis, congenital slow channel syndrome, Alzheimer's disease and epilepsy. Understanding the relationship between the structure and function of the acetylcholine receptor is necessary to explain the normal and pathologic functioning of the nervous system. Molecular biological techniques have allowed us to study the structure of the acetylcholine receptor at the level of its amino acid sequence. However, the relationship between the amino acid sequence of the receptor and its function remains controversial. Our research has focused on this relationship. In particular, we have examined the structural basis of conductance and gating of the acetylcholine receptor.

Acetylcholine receptors belong to a receptor super-family including GABA, glycine and glutamate receptors. All three of these receptors undergo a significant functional change from long to brief open time during both development (Schuetze and Role, 1987; Kidokoro, 1994). Both structural changes and post-translational modification of the receptor structure have been implicated in producing this change. Whereas the functional significance of this shift from long to brief open time is unknown, it has

been shown to alter synaptic current and play a significant role in synapse development (Owens and Kullberg, 1989). The fact that this mechanism is conserved in multiple receptor classes, as well as in mammalian and amphibian species, emphasizes the importance of understanding the principles underlying these mechanisms.

Basic physiology of receptors in the nervous system

Receptors are protein structures involved in cellular communication in the nervous system. Receptors relay information by transferring signals between cells. The site where a presynaptic cell interfaces with target cell is called a synapse. The term, 'synapse,' is derived from ancient Greek and means coming together or close apposition. The term has become more specific and now refers specifically to the interface between a neuron and a target cell, where information is transmitted from one cell to the other. The one consistent feature which is common to all chemical synapses is that the presynaptic neuron releases neurotransmitter, and the postsynaptic cell bears receptors which respond to the transmitter. In some cases, the postsynaptic receptors contain an integral ion channel, such as the case with the nicotinic acetylcholine receptor channel at the neuromuscular junction. The nicotinic acetylcholine receptor is an example of an ionotropic receptor in which both the receptor and channel are contained within a single protein structure. A second general class of postsynaptic receptors found at chemical synapses are those which do

not contain an integral ion channel, but instead are linked to internal second messenger systems within the cell. These types of receptors are often referred to as metabotropic receptors. Examples of metabotropic receptors include the α- and β-adrenergic receptors, and the muscarinic acetylcholine receptor.

The nicotinic acetylcholine receptor is activated by the binding of the neurotransmitter molecule (i.e. the ligand) to the receptor complex. The neurotransmitters that activate postsynaptic ion channels are generally small rapidly diffusing molecules bearing a highly polar or charged group. Examples of neurotransmitters include acetylcholine, glycine, glutamate, serotonin and GABA. Once activated, the receptor protein undergoes a conformational change. This change results in the opening of the channel pore through which ions flow. The resulting ion flux results in the post-synaptic cell becoming either excited or inhibited, depending on which ions permeate the particular synapse. Figure 1 illustrates activation and subsequent opening of the acetylcholine receptor channel by the binding of acetylcholine. Note that the acetylcholine transmitters bind to a cleft region between the α subunit and its adjacent γ subunit. In the case of the adult receptor, an ε subunit would be present in place of the γ subunit.

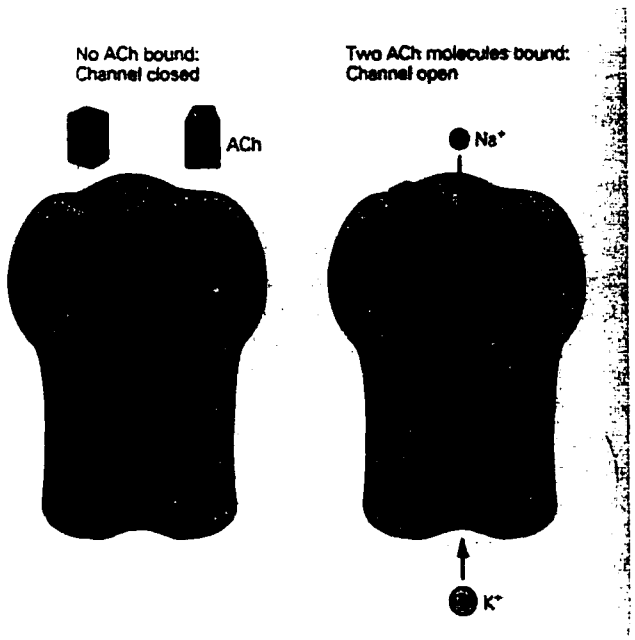

Figure 1 – The acetylcholine receptor channel (Kandel *et al.*, 2000). When two acetylcholine transmitters bind, the channel undergoes a conformational change resulting in the opening of its pore and subsequent ion flux.

At the neuromuscular junction, acetylcholine receptor activation results in a net influx of cations and hence depolarization. The cumulative charge passing through all activated receptors results in the synaptic current. The synaptic current triggers an action potential, which in turn results in the contraction of the innervated muscle. Factors that affect synaptic current will therefore influence signal transmission at the neuromuscular junction.

Biophysical properties of ion channels

Two principal aspects of channel function are conductance and gating. These two features determine how much charge passes through a channel once it has been activated by a neurotransmitter. The net change in charge across the membrane results in either the excitation or inhibition of the cell.

Conductance

Conductance refers to the ease with which charge flows through a receptor channel when opened. Conductance is the reciprocal of resistance and is related to current and voltage by Ohm's law. Ohm's law states that g=I/V, where g is the conductance, I is the current passing through the open channel and V is the voltage across the channel. In our studies, we measured the conductance in picosiemens (pS). In the case of the acetylcholine receptor, sodium and potassium ions are the charge carriers through the open channel pore. Regulating the amount of current flowing through a channel is a key mechanism of modulating neuronal or muscle excitability. Conductance can be altered by changing the size of the channel pore or by changing the net charge of amino acids surrounding or within the pore.

In both mammalian and amphibian acetylcholine receptors, a similar and significant conductance change occurs during development. The embryonic form of the acetylcholine receptor exhibits a low conductance channel (Mishina *et al.*, 1986). During development, the embryonic receptors are gradually replaced by adult

receptors having a 50% greater conductance. This change has been shown to be the result of a structural subunit substitution that naturally occurs during development from the $\alpha_2\beta\delta\gamma$ embryonic form, to the $\alpha_2\beta\delta\varepsilon$ adult form (Mishina *et al.*, 1986). Figure 2 shows a sample single channel recording illustrating the conductance difference between embryonic(γ) and adult(ε) *Xenopus* acetylcholine receptors.

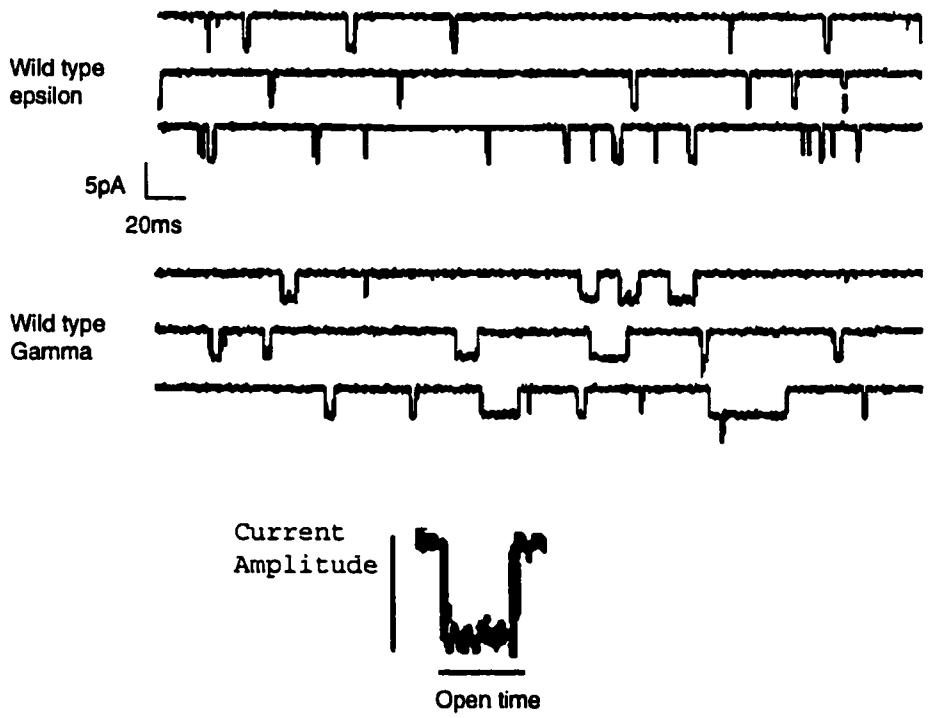

Figure 2 –Single channel recording of embryonic γ(wt) and adult ε(wt) receptors. Note the higher current amplitude and faster openings of the adult channels. The applied membrane potential is the same in the upper and lower records (-100 mV), so that the difference in current amplitude is proportional to the difference in channel conductance.

Gating

Gating refers to the mechanisms underlying opening and closure of the ion channel. One of the key elements of gating is the channel open time. The channel open time refers to the length of time that a channel stays open following activation. In the acetylcholine receptor, the gating is commonly referred to as the "open time." The gating of the nicotinic acetylcholine receptor can most easily be explained by the following diagram where **A** refers to agonist (i.e. acetylcholine), **R** refers to the receptor (i.e. nicotinic acetylcholine receptor) and **AR** refers to the agonist/receptor complex:

$$\mathbf{2A + R \Leftrightarrow A + AR \Leftrightarrow A_2R(closed) \Leftrightarrow A_2R(open)}$$

The first two steps represent the agonist-receptor binding steps. The next step represents the conformational change between the open and closed state. The open time refers to the length of time that the receptor stays in the A_2R open state. It is this measurement that we are attempting to obtain during single channel recording. However, because of bandwidth limitations in our recording system, an apparent single opening of a channel may in fact represent a burst of brief, unresolved openings, reflecting a series of rapid conformational changes between the A_2R open state and the A_2R closed state. In other words, our recording system may not be fast enough to elucidate rapid fluctuation between the A_2R open state and the A_2R closed state. For this reason, in single channel recording, the 'channel open time' is often

referred to as the 'burst duration' to account for the inherent possibility that we are actually observing a series of conformational changes between A_2R open and the A_2R closed rather than a single, prolonged opening. In this thesis, the term 'open time' is used to signify apparent single openings, some of which may in fact be bursts of opening separated by unresolved closures.

As with conductance, there is a developmental change in open time that occurs during synapse maturation. The embryonic($\alpha_2\beta\delta\gamma$) receptor has a long open time compared to the relatively short open time in the adult($\alpha_2\beta\delta\varepsilon$) receptor (Owens and Kullberg, 1989; Bouzat *et al.*, 1994). Channel open time is also thought to be influenced by multiple mechanisms. Changes in subunit composition of the receptor and post-translational modification of the receptor are both thought to play important roles regulating the channel open time (Owens and Kullberg, 1993; Lu *et al.*, 1993).

Basic acetylcholine receptor anatomy

The acetylcholine receptor channel is formed from the assembly of 5 constituent subunit proteins. Each subunit has a similar secondary and tertiary structure comprising four membrane-spanning regions as well as intracellular and extracellular loops (See figure 3).

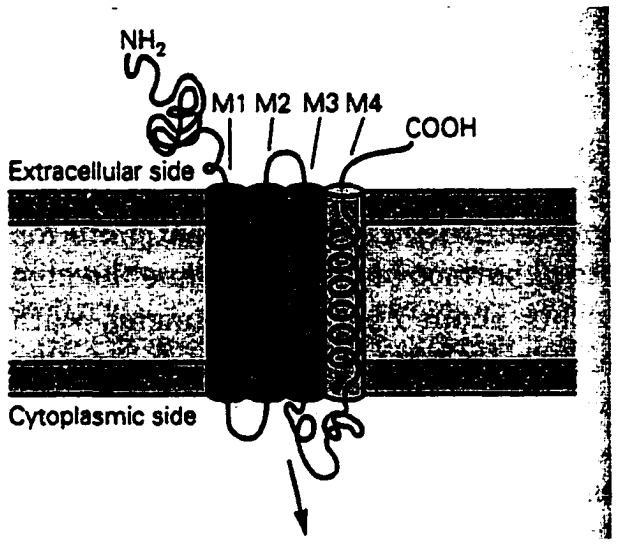

Figure 3 – A single subunit of the acetylcholine receptor (Kandel *et al.* 2000). The M1 through M4 regions are the membrane spanning regions of the receptor.

The subunits assemble into a tertiary structure, which results in a central pore channel through which ions flow when the receptor is activated. The amphibian and mammalian acetylcholine receptors exhibit an identical subunit configuration. There are 5 distinct but homologous subunits named alpha(α), beta(β), gamma(γ), delta(δ) and epsilon(ε). Figure 4 illustrates how the 5 subunits assemble to form a channel.

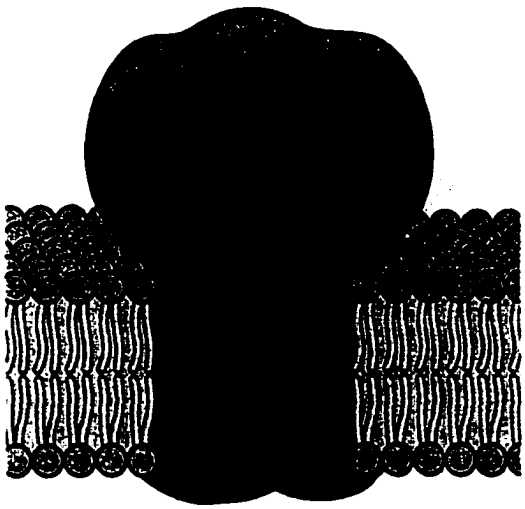

Figure 4 - Tertiary structure of nicotinic acetylcholine receptor (Kandel et al. 2000)

M2 region anatomy

Assuming that the change in receptor function of the acetylcholine receptor during development was due to the subunit compositional change from γ to ε, we hypothesized that the M2 region would be the most likely region to account for the changes in conductance. The reason for choosing the M2 region was due to its location within the channel pore. Figure 5 illustrates the arrangement of the subunits into a channel. It shows that the M2 region of each subunit faces the channel pore. For this reason we chose the M2 region of γ and ε subunits as a target for experimental mutations. Figure 6 lists the amino acid sequences of the Xenopus γ and ε subunit M2

regions. The figure also illustrates the homology with the mammalian (i.e. rat) γ and ε subunits. The boxed residues indicate differences between the two subunits.

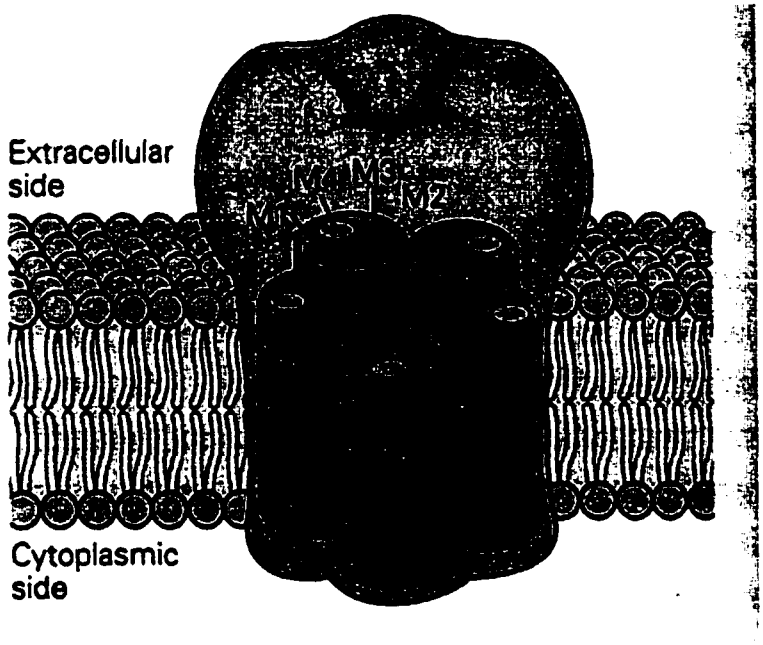

Figure 5 – The arrangement of subunits into a channel (Kandel *et al.* 2000). **Note that the M2 region faces the channel pore.**

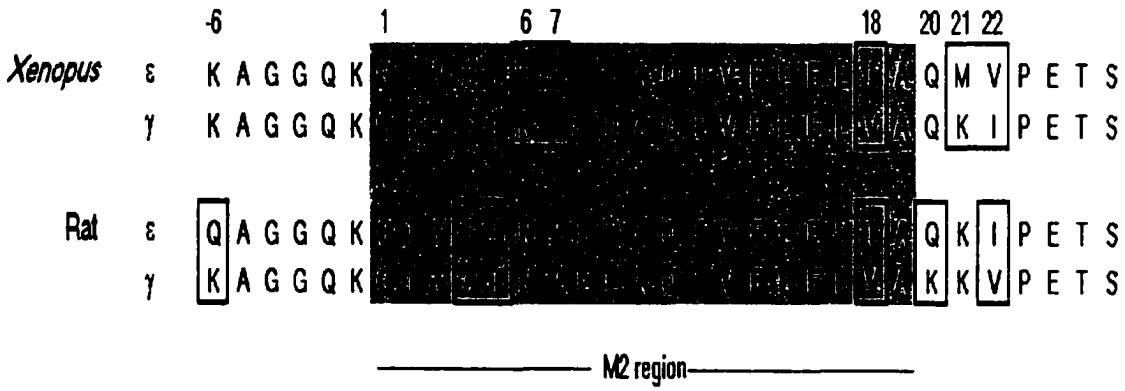

Figure 6 – Alignment of M2 regions of *Xenopus* and rat ε and γ-subunits. Boxed residues indicate differences between the embryonic and adult subunits within a species. Numbering begins with the first residue of the M2 region.

Experimental Design

We hypothesized that the non-conservative amino acid substitution of serine for asparagine at position six was a likely modifier of receptor channel conductance. The residues at position six, as well as certain other amino acid residues in and flanking the M2 region, were investigated.

In our experiments, we genetically manipulated residues of the adult and embryonic *Xenopus laevis* (South African claw frog) acetylcholine receptor by performing a series of point mutations. We then studied these changes by expressing these mutant channels in *Xenopus* oocytes. The electrophysiological technique of patch clamp single channel recording (Neher and Sakmann, 1976) allowed us to study the electrophysiological characteristics of individual acetylcholine receptor channels. We used single channel recording techniques to obtain quantitative measurements of conductance and open time data for several acetylcholine receptor mutants as well as wild-type(wt) receptors. These studies resulted in a significant contribution to our understanding of which amino acid residues produce the developmental shift in open time and single channel conductance of the acetylcholine receptor at the neuromuscular junction.

In addition to studying structural determinants of channel open time, we also studied the effects of post-translational modification on channel open time. Post-translational modification of the channel, especially phosphorylation, has been shown to play an important role in channel open time (Fu and Lin, 1993; Owens and Kullberg, 1993). To examine the effects of phosphorylation on channel open time, we performed experiments using phosphorylating agents (okadaic acid and 8-bromo cAMP). In addition, we performed mutations of the protein kinase A phosphorylation sites on the alpha, beta, delta and gamma subunit. These mutations prevented PKA

phosphorylation and allowed us to examine the role phosphorylation may play in regulating channel open time.

The open time of the receptor channel and the conductance of the channel when open are two properties of acetylcholine receptor function that are critical in the proper functioning of the synapse. This thesis explores the structure-function relationship of the nicotinic acetylcholine receptor in amphibian muscle. The studies reported here were carried out on *Xenopus laevis* embryonic and adult acetylcholine receptors. The results of these experiments are compared to reported data from mammalian species in order to help explain the different structural mechanisms by which mammals and amphibians achieve similar functional changes in conductance and gating during development.

CHAPTER 2
METHODS

Experimental Animals

Xenopus laevis frogs were the amphibian species used for this experiment. *Xenopus* frogs are extremely amenable to experimental work due to their availability, ease of care, resistance to infection and well-documented development. Adult *Xenopus* females obtained from NASCO (Fort Atkinson, WI) were used in all of our experiments. All work with animals was conducted in accordance with NIH guidelines and under the supervision of the institutional animal care and use committee.

Molecular Techniques

Standard molecular techniques were used for all DNA and RNA preparation and manipulation (Sambrook *et al.*, 1989). cDNA clones encoding *Xenopus* acetylcholine receptor α, β, δ, γ and ε subunits were used for these experiments. Oligonucleotides were designed for site directed mutagenesis and then ordered from Oligo's Etc. (Bethel, ME). Site directed mutagenesis of γ and ε subunits was performed using either the Morph Mutagenesis Kit (5 Prime $\rightarrow$ 3 Prime, Inc., Boulder, CO), or the method of Kunkel (Sambrook *et al.*, 1989). Mutant clones were confirmed via dideoxy sequencing of mutant strains.

The mutations performed in these studies exclusively involved replacing epsilon residues with their gamma counterparts, or vice versa. I performed all mutations in our laboratory with the exception of the ε(HA) chimera, which was supplied to us by the laboratory of Paul Brehm (SUNY at Stonybrook). The eleven different experimental cRNAs in addition to the ε(wt) and γ(wt) controls are listed below in Table 1. The abbreviation and explanation of each experimental preparation are also listed in the Table. All expressed receptors had identical α,β, and δ subunits. Subunit cRNAs were prepared from cDNA using Ambion mMessage mMachine SP6 and T3 kits (Austin, TX). The transcripts were resuspended in nuclease-free water at a concentration of 200 ng/μl (α subunit transcript) or 100 ng/μl (β, δ, γ and ε subunit transcripts). Samples were aliquoted and stored at -70°C until used. Stock solutions of each type of subunit cRNA were premixed to create injection solutions (α 50 ng/ml, β 25 ng/ml, δ 25 ng/ml, γ or ε 25 ng/ml).

Table 1 – Abbreviations and Descriptions for all Constructs

γ(wt)	gamma wild type receptor
ε(wt)	epsilon wild type receptor
γ(n6s)	a gamma wild type receptor with site 6 in the M2 region mutated from asparagine to serine.
ε(s6n)	an epsilon wild type with site 6 mutated from serine to asparagine.
γ(n6s v7i)	γ(wt) with site 6 mutated from asparagine to serine and site 7 mutated from valine to isoleucine.
ε(s6n i7v)	ε(wt) with site 6 mutated from serine to asparagine and site 7 mutated from isoleucine to valine
γ(εHA)	γ(wt) with the entire HA region identical to that of epsilon's. Note that the HA region is an amphipathic helix located in the M3-M4 intracellular linker (See Figure 3).
γ(εHA n6s)	γ(wt) with the entire HA region identical to that of epsilon's and site 6 mutated from asparagine to serine.
γ(εm2f)*	γ(wt) with site 6 mutated from asparagine to serine, site 7 mutated from valine to isoleucine, site 18 mutated from valine to isoleucine, site 21 mutated from lysine to methionine and site 22 mutated from isoleucine to valine.
ε(γm2f)*	ε(wt) with site 6 mutated from serine to asparagine, site 7 mutated from isoleucine to valine, site 18 mutated from isoleucine to valine, site 21 mutated from methionine to lysine and site 22 mutated from valine to isoleucine.
γ(s>a)	γ(wt) with the PKA phosphorylation site at position 371 of the γ subunit mutated from serine to alanine.

*** The f refers to the M2 flanking regions (i.e. site 18, 21 and 22) that lie outside of the M2 region but have been shown to influence channel open time, (Murray *et al.*, 1995).**

Oocyte Preparation

Oocytes were surgically excised from freshly euthanized *Xenopus* frogs. Euthanasia was performed by ice bath immersion anesthesia followed by a blow to the head. The follicular membrane was removed from the oocyte by microdisection. To facilitate microdisection, the oocytes were soaked in a 15mg/ml solution of collagenase for 8-15 minutes. Following defolliculation, the oocytes were stored in an OR3 solution (See Table 2) at 17° C pending injection.

Table 2 - Solutions

OR3

(50% Gibco L-15 media, 15 mM HEPES, 1 mM L-glutamate, 20 mg/ml gentamicin, adjusted to pH 7.6). This nutrient solution was used for the storage of oocytes prior to single channel recording

Ringer solution

(120 mM NaCl, 1 mM KCl, 1.8 mM $CaCl_2$, 1 mM $MgCl_2$, and 10 mM HEPES, adjusted to pH 7.4). This solution was the base solution used rinsing of oocytes. It also served as the recording solution for the PKA phosphorylation experiments.

Recording solution

(120 mM NaCl, 1 mM KCl, 0.4 mM $CaCl_2$, 1 mM $MgCl_2$, and 10 mM HEPES, adjusted to pH 7.4). With the exception of the PKA experiments, all recordings were obtained using this solution.

Pipette solution

(80 mM KF, 20 mM KCl,10 mM K-EGTA, 10 mM HEPES, adjusted to pH 7.4). This solution was used to fill the recording pipette.

Oocyte Injection

Oocytes were injected with cRNA with a Medical Systems PLI-100 injector. Injection pipettes were pulled from capillary tubes and beveled to an outer diameter of 18-20 μm. Pipettes were baked at 200° C for 2 hours to eliminate RNase contamination. All pipettes were stored in a metal box pending injection. Each oocyte was injected with 50 nl of cRNA.

Phosphorylating Agents

For phosphorylation experiments, okadaic acid and 8-bromo-cAMP were used to increase likelihood of phosphorylation. Oocytes used for phosphorylation recording were placed in an OR-3 solution containing 2μm okadaic acid and 500μm 8-bromo-cAMP the evening prior to recording. The oocytes were exposed to phosphorylating agents for 12-18 hours prior to recording.

Single Channel Recording

Patch electrodes were pulled to an outer diameter of 1-2 μm and then fire polished. Just before recording, each electrode was filled with pipette solution and the tip was coated with Sigmacote (Sigma, St. Louis, MO). The electrode was then ready for recording and was placed in the single channel recording apparatus.

Oocytes were allowed to incubate for 2 days prior to recording to allow for expression of the acetylcholine receptors. Immediately prior to recording, oocytes were immersed in a solution of 0.05% trypsin-EDTA to help facilitate removal of the vitelline membrane. Following 90 seconds of immersion the oocyte was thoroughly rinsed in Ringer's solution and the vitelline membrane was removed by microdisection. The oocyte was then placed in a holding chamber adjacent to the recording chamber.

Outside-out patches were excised from the oocyte membrane and transferred to the recording chamber. The solution level was lowered to prevent recording solution from entering and contaminating the adjacent oocyte. The volume of the recording trough was 0.7 ml. and the solution flow rate was 3-4 mL min^{-1} .

Patches were exposed to recording solution for at least one minute to allow for complete washout of Ringer solution before recording commenced. Washout was essential due to higher Ca^{2+} concentration (1.8 mM) in the Ringer solution than in the recording solution (0.4 mM). The long period of washout prevented possible effects of residual calcium on channel conductance. For the PKA experiments, all recordings were performed in ringers solution.

All data were obtained from outside-out patches in recording solution with concentrations of acetylcholine ranging from 125 to 500 nM. Single channel events

were recorded by a patch amplifier (EPC-7), filtered at 3 kHz, and continuously digitized at 10 kHz with an ITC-16 A/D converter (Instrutech Inc.). For kinetic analysis, records were acquired at a continuous applied pipette potential of −100 mV. For current/voltage data, the pipette potential was stepped through potentials from −60 to −160, in 20 mV steps, with 8 seconds of data acquired at each step.

Analysis

Digitized records were individually analyzed with MAC-TAC analysis software (Skalar Instruments). Prior to kinetic analysis, records were digitally filtered at 2683 Hz, giving an overall corner frequency of 2000 Hz. For the detection of channel openings, an amplitude threshold was set at about ½ full amplitude. Overlapping events were excluded from the analysis. The burst resolution was set at 0.3 ms, and for the purposes of this paper, the term "open time" refers to the burst duration. For kinetic analysis, the average analyzed sample size from each recording site was more than 500 events, and the minimum size was 100 events. The durations of brief events were corrected for the corner frequency of 2000 Hz. Log-open time histograms were fitted with the sum of two exponential functions and the weighted mean channel open time of each histogram was calculated from the two exponential components.

For conductance analysis, records were digitally filtered at either 2683 Hz or 1000 Hz. The frequency response of the recording system was not crucial in measuring amplitudes since only events that clearly reached full amplitude were used for I/V

analysis. I/V plots were generated by measuring the amplitudes of events acquired at successive potentials from –60 mV to –160 mV. Typically 5-15 events were measured at each potential. Some patches exhibited more than one class of channel amplitudes. When low amplitude events were present, they were usually a minority class and in such cases, we confined our measurements to the class of events with the largest amplitude. The individual I/V plots corresponding to each construct were pooled and the resulting average I/V plot was fitted with a quadratic polynomial. The derivative of the fitted curve was used to estimate the slope conductance at –100 mV.

CHAPTER 3
RESULTS

Open Time

Effects of mutations at positions 6 and 7 on open time

Our results showed that acetylcholine receptors having the embryonic γ(wt) composition exhibit an open time that is about 3-fold longer than that of receptors having the adult ε(wt) composition. We found that the sites 6 and 7 of the pore-forming M2 domain account for the major change in open time that occurs during development. The data show that the long open time embryonic γ(wt) channel can be almost completely converted to the short open time adult ε(wt) channel by substituting a single amino acid residue at position 6. By adding the mutation at position 7, the open time is virtually identical to that of the adult receptor. Figure 7 illustrates single channel recording data for the four main subunit classes in our open time experiments.

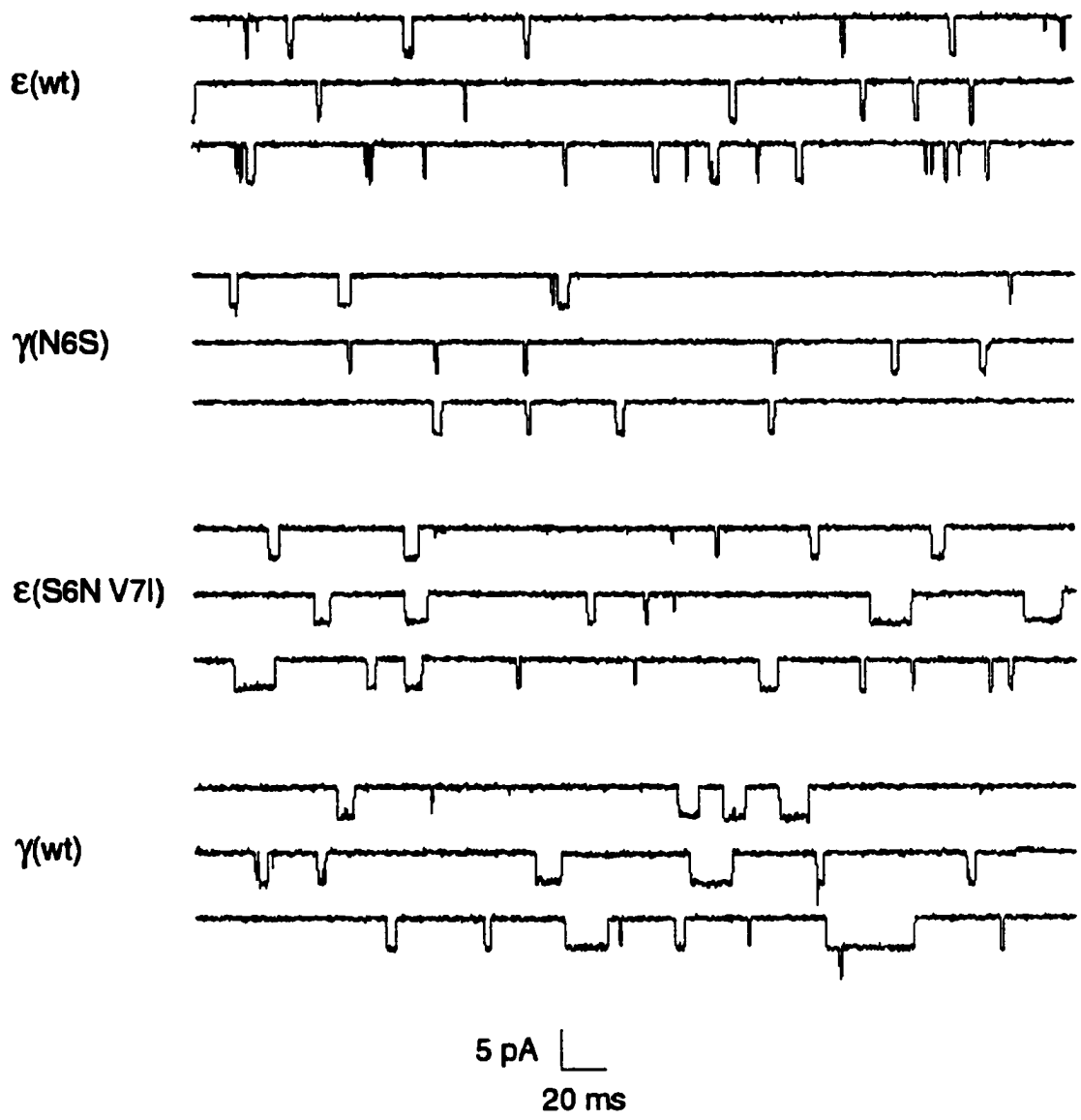

Figure 7 – Single channel records obtained from wild type and mutated receptors. The applied membrane potential was –100 mV. Note the brief open time of the ε(wt) and γ(N6S) vs. the γ(wt) and ε(S6N V7I).

As explained in the Methods, an open duration histogram was compiled from the measured channel open times of each recording and fitted with a double exponential function. For each histogram, the weighted mean open time was computed from the double exponential parameters and employed as a simple means of comparing the effects of different mutations. Example histograms are shown in figure 8.

The data showed that the ε(s6n) mutation increased the mean open time by 1.7 fold. When both positions 6 and 7 in ε were mutated to their counterparts in γ, (ε s6n v7i), the mean channel open time increased 3.5 fold. The resultant mean open time was not significantly different from that of wild type γ acetylcholine receptor given the large variability in open time characteristic of these channels.

The converse mutation of γ(n6s) resulted in a 2.5-fold reduction in mean open time. This reduction resulted in the γ(n6s) mutant having a comparable open time to the ε(wt) channel. An additional mutation of position 7 from isoleucine to valine γ(n6s i7v) resulted in no further significant reduction of open time. Examination of the double exponential parameters indicates that the major effect of position 6 and 7 mutations on open time histograms was on the time constant of the slow component. There was considerable variability in the fast component and in the areas of the two components, but these parameters exhibited no trend comparable to that of the slow component. Figure 9 illustrates the effects of the M2 mutations on double exponential parameters.

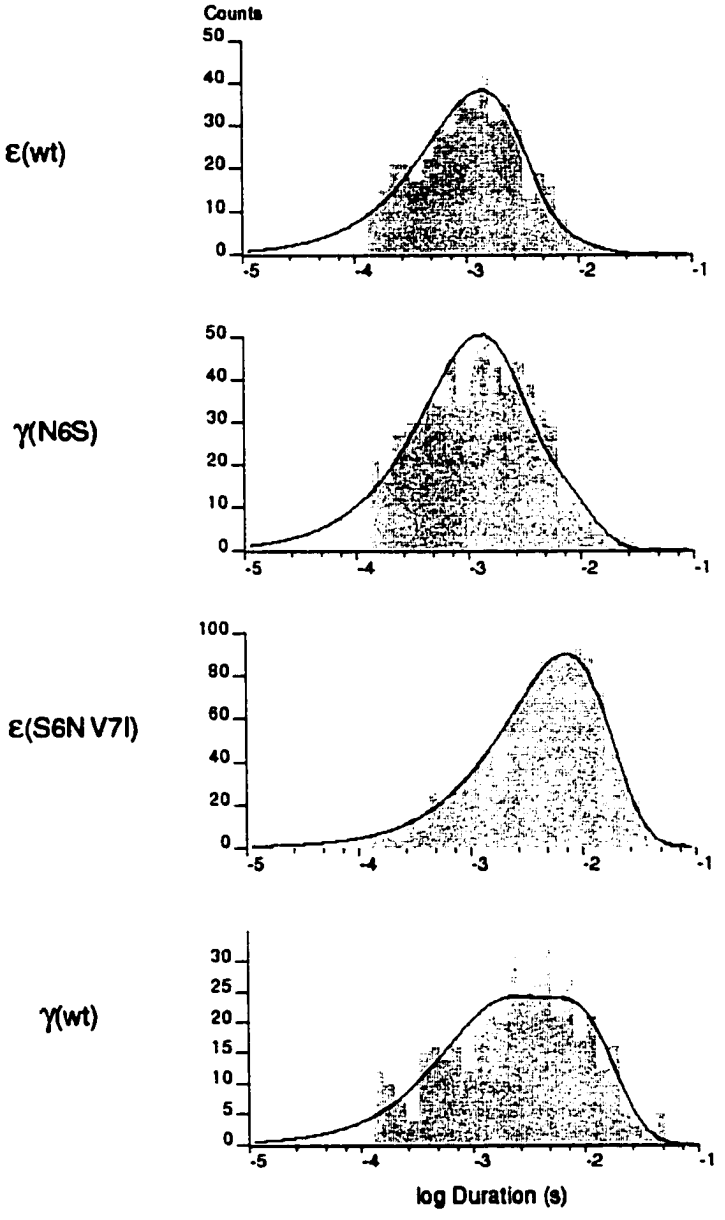

Figure 8 – Open duration histograms of single channel events.
This figure illustrates the effects of M2 mutations of channel open time. The curves are fitted with double exponential functions, which were used to compute the following weighted mean open times: ε (wt), 1.73 ms; γ (N6S), 2.09 ms; ε (S6N V7I), 7.28 ms; γ (wt), 5.36 ms.

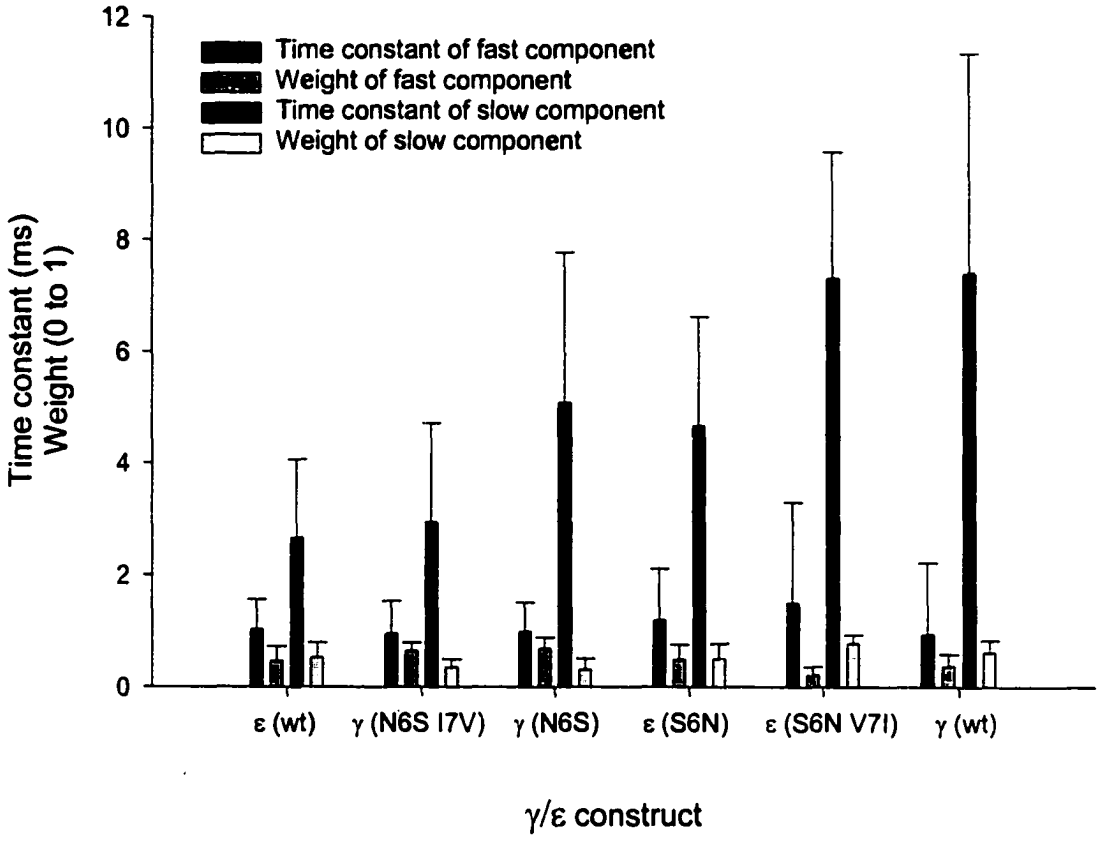

Figure 9 - Effect of γ and ε M2 mutations on double exponential parameters. **Individual open duration histograms were fitted with double exponential functions by the method of maximum likelihood. The estimated parameters from several recordings of each construct were averaged to produce the data above. Each bar shows the mean parameter ± s.d. These data were used to compute the weighted mean open times in Figure 10. Table 3 lists the individual single channel records used for this figure including sample size and standard deviation for each group.**

TABLE 3

Composition	Average tau 1	sd tau 1	Average area tau1	sd area tau1	Average tau 2	sd tau 2	Average area tau2	sd area tau2	Sample size
[illegible]	0.95	0.68	0.65	0.45	2.95	1.97	0.35	[illegible]	[illegible]
αβδ(wt) ε(wt)	1.03	0.53	0.47	0.26	2.65	1.41	0.53	0.26	23
[illegible]	1.19	0.92	0.49	0.37	4.66	1.97	0.51	[illegible]	[illegible]
[illegible] γ(n6s)	0.99	0.52	0.69	0.20	5.08	2.69	0.31	0.20	12
[illegible]	1.50	1.80	0.22	0.15	7.32	2.26	0.78	[illegible]	[illegible]
αβδ(wt) γ(wt)	0.95	1.28	0.38	0.22	7.41	3.94	0.62	0.22	20

Table 3 - Effect of gamma and epsilon M2 mutations on double exponential parameters.

The data suggests that the developmental decrease in acetylcholine receptor channel open time, associated with the substitution of an ε subunit in place of a γ subunit, depends on the changes in residues in positions 6 and 7 of the M2 region. When these changes were eliminated in the ε subunit, channels containing that subunit no longer exhibited the brief open times characteristic of adult acetylcholine channels. A summary of the effects of the M2 region mutations is illustrated in figure 10.

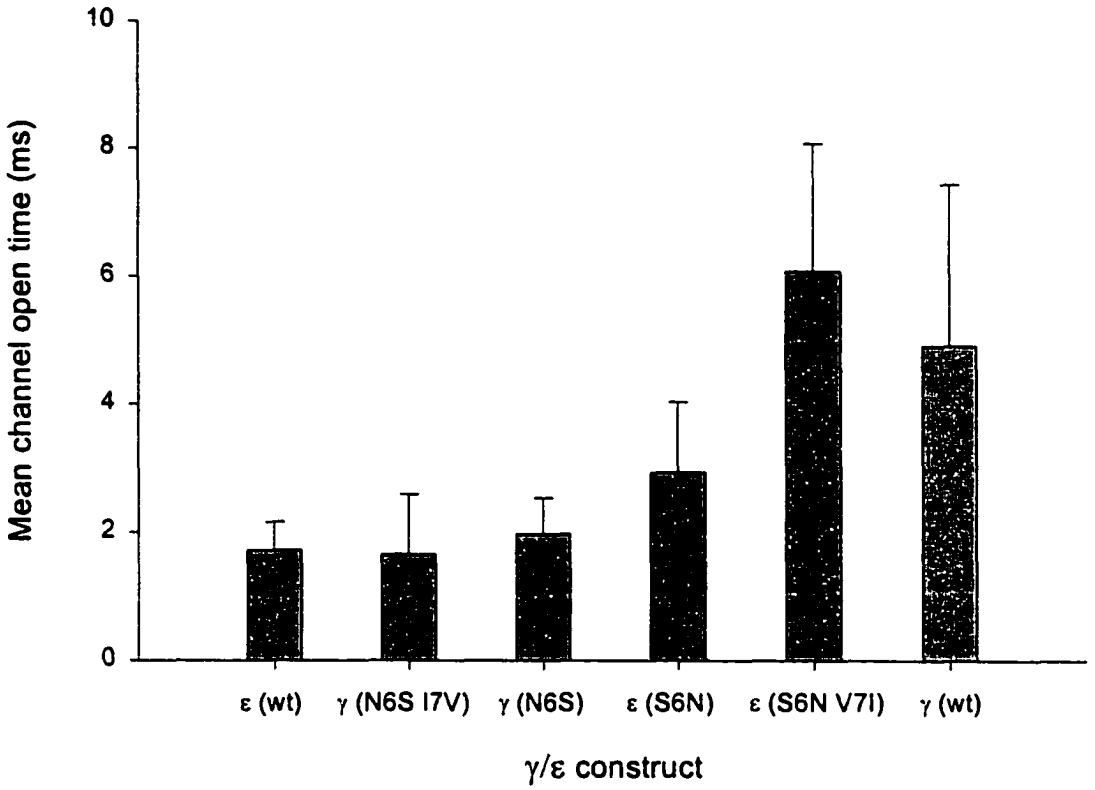

Figure 10 - Effect of M2 mutations on channel open time. **Each bar indicates the weighted mean channel open time obtained from several recording sites for each of the constructs listed tested. The double exponential parameters used to compute the weighted mean open times are summarized in figure 9. Data were obtained at –100 mV. The weighted mean channel open times (± s.d.) and sample sizes presented above are as follows: ε (wt), 1.72 ms ± 0.44 (23); γ (N6S I7V), 1.65 ms ± 0.94 (12); γ (N6S), 1.97 ms ± 0.57 (12); ε (S6N), 2.94 ms ± 1.09 (14); ε (S6N V7I), 6.08 ms ±1.99 (16); γ (wt) 4.91 ms ± 2.52 (20). The difference between the open times of ε (S6N V7I) and γ (wt) was not statistically significant (two-tailed t test, P = 0.092). The difference between the open times of ε (wt), γ (N6S I7V) and γ (N6S) were not statistically significant (two-tailed t test, P > .05).**

Effects of M2 flanking domain on channel open time

In order to determine if the residues at positions 18, 21 and 22, which differed between γ and ε subunits, contributed to the change in open time that occurs during development, we studied mutations at these regions as well. Figure 11 illustrates the mean channel open time for the αβδ(wt)ε(γm2f) mutant versus the αβδ(wt)ε(s6n v7i) mutation described above. This data shows that the M2 flanking regions have no additional effect on channel open time.

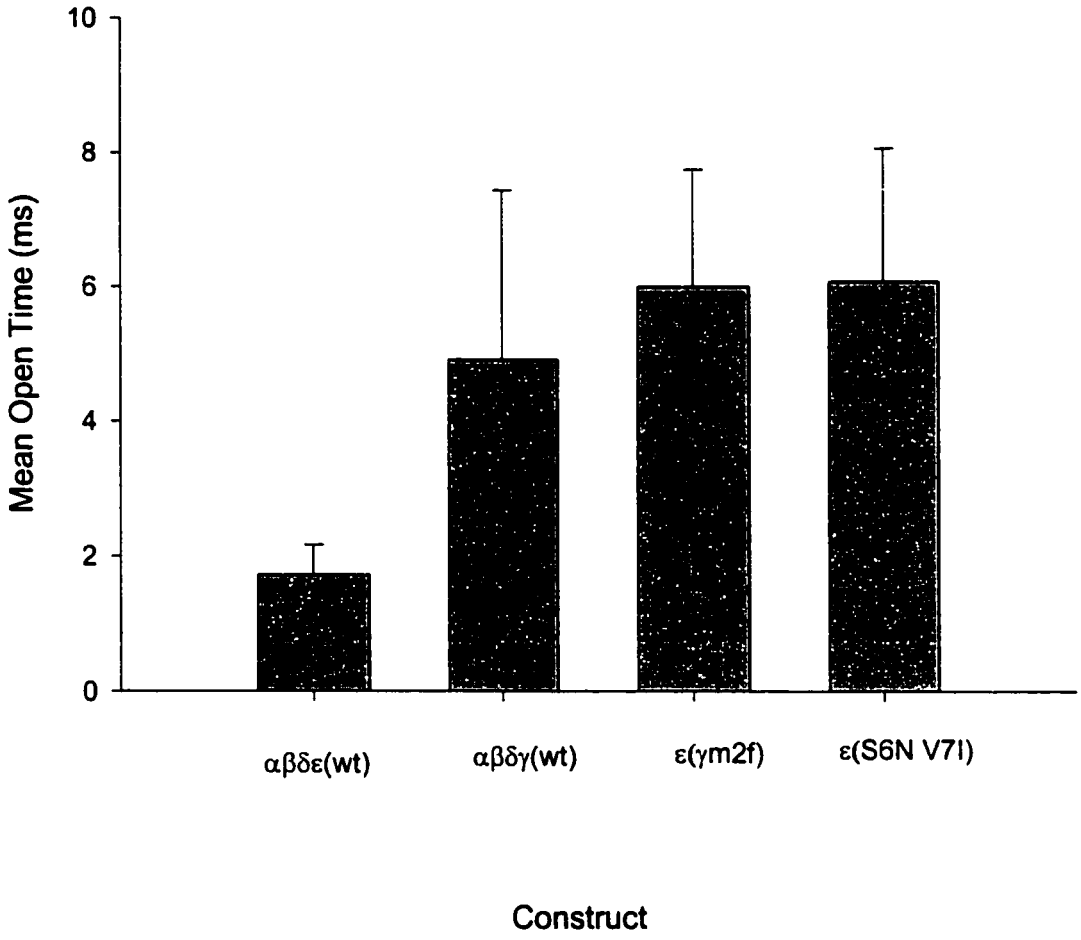

Figure 11 – Effects of ε M2 flanking regions on channel open time. **Each bar indicates the weighted mean channel open time obtained from several recording sites for each of the constructs listed tested. Data were obtained at – 100 mV. The weighted mean channel open times (± s.d.) and sample sizes presented above are as follows: ε (wt), 1.72 ms ± 0.44 (23); γ (wt) 4.91 ms ± 2.52 (20); ε (γm2f), 6.00 ms ± 1.74 (9); ε (S6N V7I), 6.08 ms ±1.99 (24). There is no statistically significant difference between the γ(wt), ε(γm2f) or ε(s6n v7i) groups (P>.05). The ε(wt) is significantly different from all groups (P<.05).**

The reverse mutation of γ(εm2f) was also studied. Unlike its converse ε version, the γ(εm2f) mutation produced channel open times that were not only faster than the γ(n6s v7i), but that were also faster than ε(wt) itself. Figure 13 illustrates the γ(εm2f) mean channel open time compared to the γ(n6s v7i) mutant.

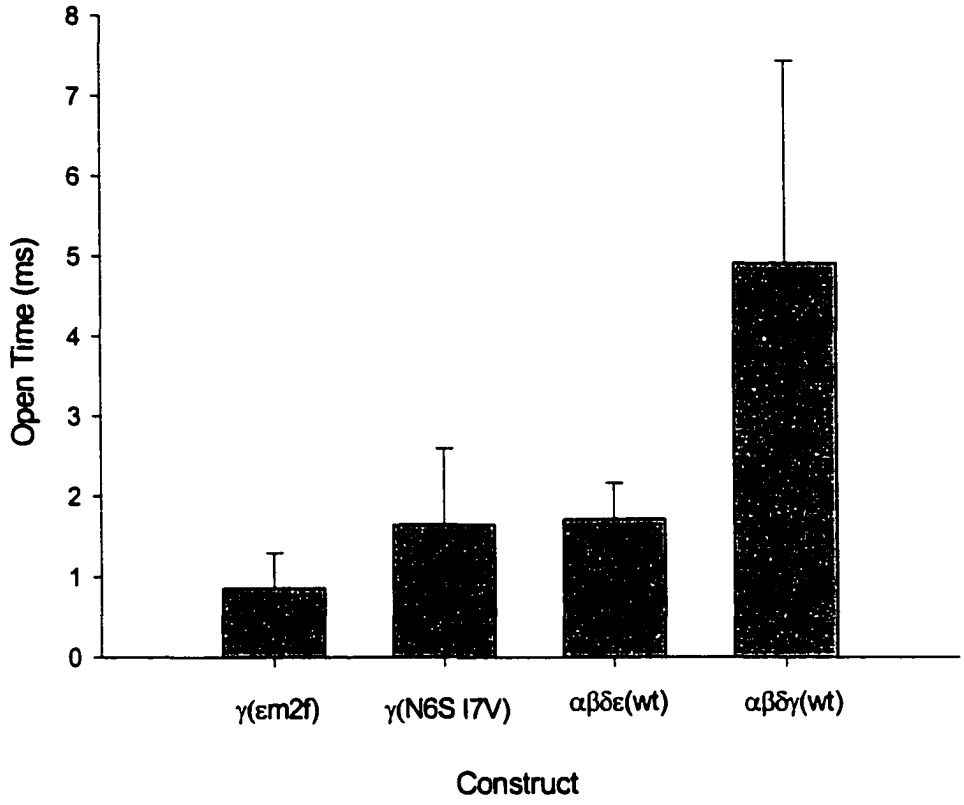

Figure 12 - Effects of γ M2 Flanking Regions on Channel Open Time.
Each bar indicates the weighted mean channel open time obtained from several recording sites for each of the constructs listed tested. Data were obtained at – 100 mV. The weighted mean channel open times (± s.d.) and sample sizes presented above are as follows: ε (wt), 1.72 ms ± 0.44 (23); γ (wt) 4.91 ms ± 2.52 (20); γ (εm2f), 0.86 ms ± 0.44 (8); γ (n6s i7v), 1.65 ms ± 0.94 (12). There is no statistically significant difference between e(wt) and γ (n6s i7v) (P>.05). The γ (εm2f) is significantly different from all groups (P<.05).

Effects of HA region on channel open time

In mammals, the amphipathic HA region has been proven to be the key region in conferring the change in open time between mammalian embryonic and adult receptors (Bouzat *et. al.*, 1994). In amphibians, as our data above have shown, it is the M2 pore-forming region that confers the change in open time. In order to examine the contribution of the HA region in amphibians, we performed single channel recording with a $\alpha\beta\delta\gamma$ chimera in which the γHA region was replaced with the εHA region. Our data, as shown in figure 13, show that the HA substitution results in a 2.3–fold decrease in channel open time. This 2.3–fold decrease brings the open time of the embryonic γ receptor almost completely down to the level of the adult ε receptor (a 3-fold decrease).

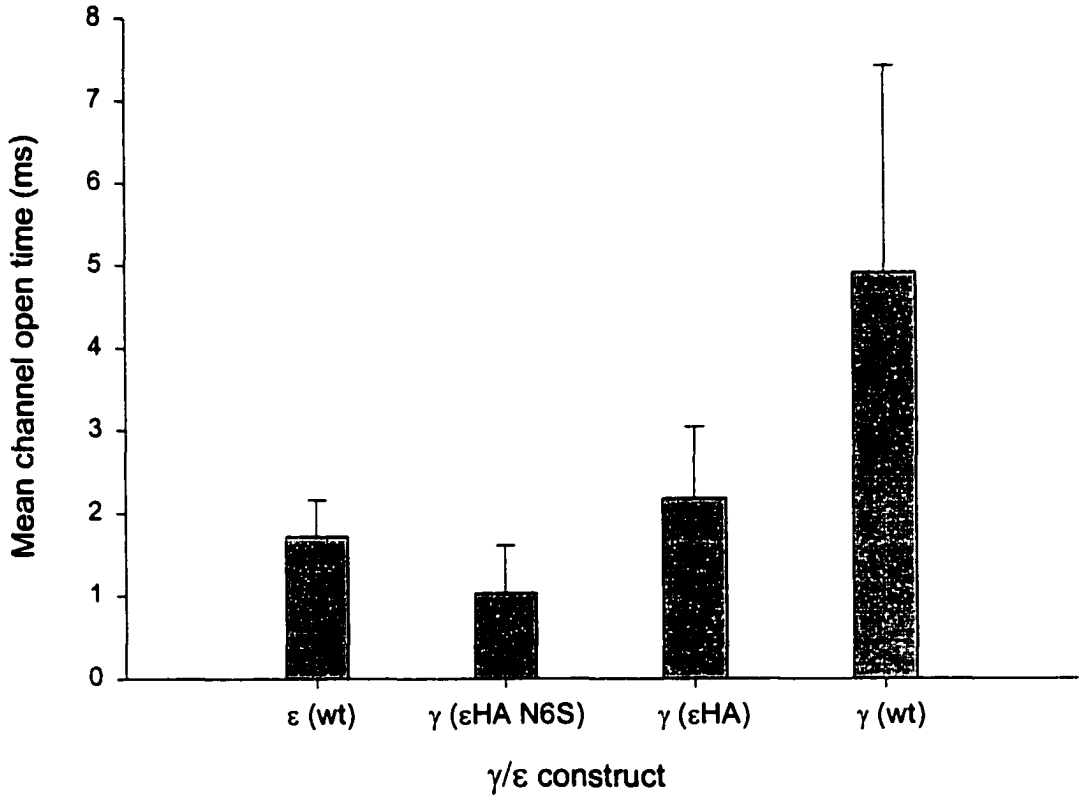

Figure 13 - Effects of HA substitution on channel open time.
Each bar indicates the weighted mean channel open time obtained from several recording sites for each of the constructs listed on the abscissa. Data were obtained at –100 mV. The weighted mean channel open times (± s.d.) and sample sizes presented above are as follows: ε (wt), 1.72 ms ± 0.44 (23); γ (εHA N6S), 1.03 ms ± 0.58 (5); γ (εHA), 2.18 ms ± 0.86 (8); γ (wt) 4.91 ms ± 2.52 (20). The mean open time of ε (wt)-AChRs is significantly less than that of γ (εHA)-AChRs (one-tailed t test, P = 0.031) and significantly greater than that of AChRs bearing the γ (εHA N6S) subunit (P = .003).

We next examined the cumulative effect of the γ(εHA) mutant with the γ(n6s) mutant predicting that the combined mutant would yield a channel open time similar to that of ε(wt). Instead we found that the HA mutation and the n6s mutation had an additive effect. The combined γ(εHA n6s) mutant had an open time of 1.03 ms, which was 40% faster than ε(wt) alone. We did not perform the reverse HA experiments. Figure 13 shows the combined mean channel open time results of the HA chimera data compared to γ(wt) and ε(wt). The results indicate that there are multiple structural regions in the amphibian acetylcholine receptor that can influence open time.

Effects of Phosphorylation on Channel Open Time

The data in Figure 14 suggest that post-translational modification was also a modulator of channel open time. We found that treatment with phosphorylating agents of the γ(wt) receptor caused a small but significant increase in channel open time. We also showed that when the PKA phosphorylation site on the gamma subunit was mutated, the resulting mutant channel had a briefer open time than the control γ(wt) channel. When this mutant channel was exposed to phosphorylating agents, the open time was unchanged. These results suggest that the phosphorylation of the embryonic γ subunit is a potential regulatory mechanism of channel open time. Figure 14 shows the phosphorylation data for the above describe experiments.

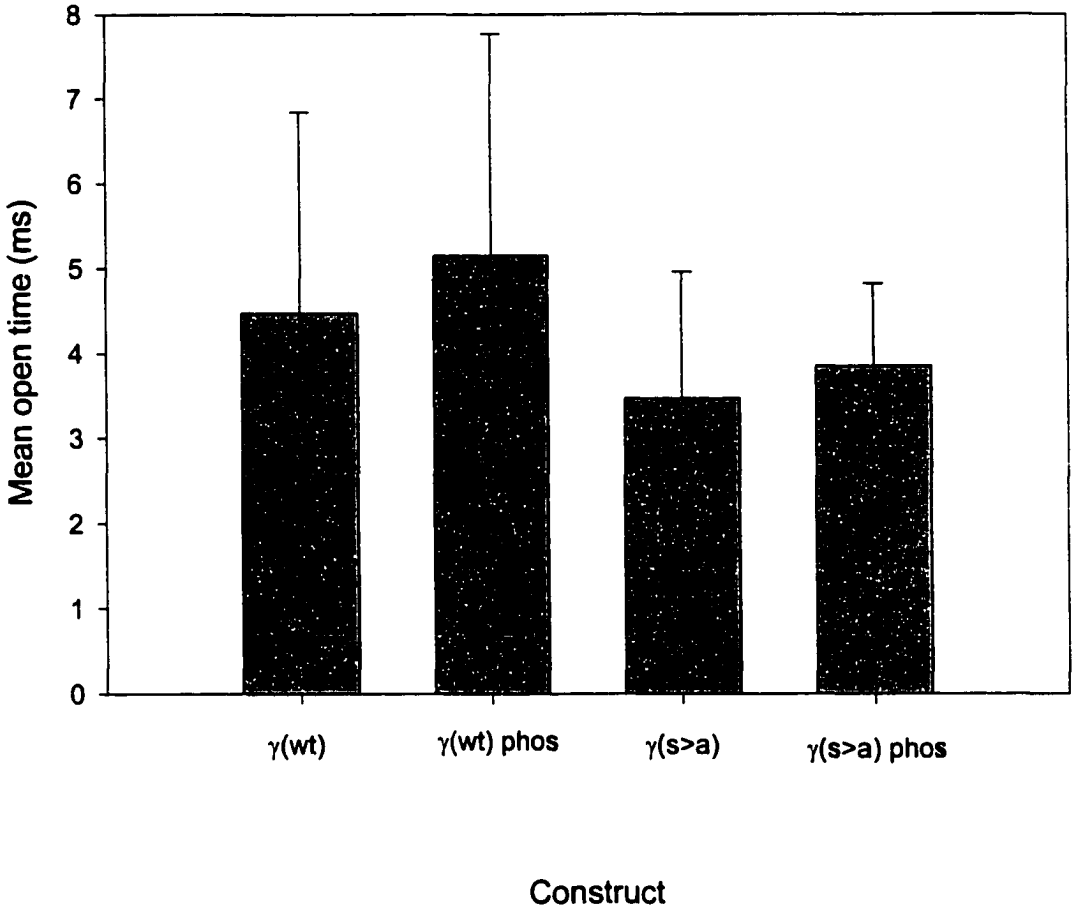

Figure 14 - Effects of Phosphorylation on Channel Open Time.
The weighted mean channel open times +/- S.D. and (sample sizes) presented above were as follows: γ(s>a) 3.47 +/- 1.49 (29); γ(wt) 4.47 +/- 2.37 (144); γ(s>a phos) 3.85 +/- 0.97 (9); γ(wt phos) 5.15 +/- 2.62 (77). All groups were statistically significantly different (P<.05) with the exception of [γ(s>a) vs. γ(s>a phos) P=0.20] and [γ(s>a phos) vs. γ(wt) P= 0.22].

Conductance

The inclusion of conductance experiments in our examination of the structure-function studies of embryonic versus adult acetylcholine receptors was serendipitous. The conductance changes from the embryonic to adult acetylcholine receptor had recently been described (Murray *et al.*, 1995). In learning the techniques of site directed mutagenesis and single channel recording, my first experiment was to attempt to confirm the conductance and open time data reported by Murray *et al.* (1995). In these experiments, I was unable to replicate the data showing that site 21 flanking the M2 region was the site responsible for conferring the conductance change during development. Furthermore, when beginning my open time experimentation on sites 6 and 7 in the M2 region, I discovered that these mutations did affect conductance. Also, neither our lab nor Dr. Murray's lab has been able to replicate γ(k21m) conductance data reported in their paper. As a result of the experiments conducted in this thesis research, the data of Murray *et al.* (1995) were formally retracted (Murray *et al.*, 1998).

Effects of M2 mutations at positions 6 and 7 on conductance

Our data showed an average slope conductance of 52 pS at −100mV for ε(wt) and 37 pS for channels for γ(wt). As figure 15 illustrates, the slope conductance of ε(wt) was significantly decreased by the s6n point mutation (45 pS). The slope conductance was further decreased by the addition of the v7i mutation (41 pS).

The converse experiments in which the γ(n6s) and γ(n6s i7v) mutants were studied revealed the expected converse result. The γ(n6s) mutation raised the slope conductance from 37.3 pS in γ(wt) to 45 pS in γ(n6s). The additional mutation of γ(n6s i7v) added little to conductance increasing it to 46 pS. Figure 16 illustrates the effect of the γ subunit mutations on channel slope conductance.

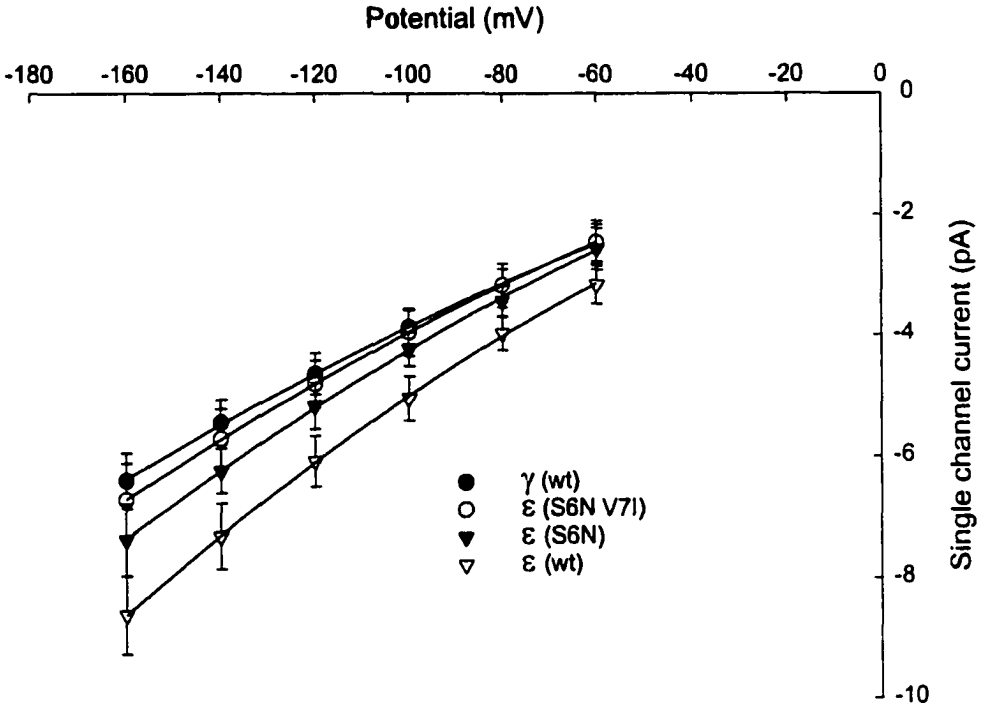

Figure 15 - Effect of ε M2 mutations on channel slope conductance. Each data point represents the mean ± s.d. channel current at a given potential for oocytes injected with wild type α, β and δ subunits plus the construct indicated in the symbol legend. The pooled current/voltage data for each construct listed were fitted by 2nd order polynomial functions. The coefficients of the fitted curves were used to compute the slope conductance at –100 mV. The estimated slope conductances were as follows: γ (wt), 37.3 pS; ε (S6N V7I), 40.8 pS; ε (S6N), 45.4 pS; ε (wt), 51.9 pS.

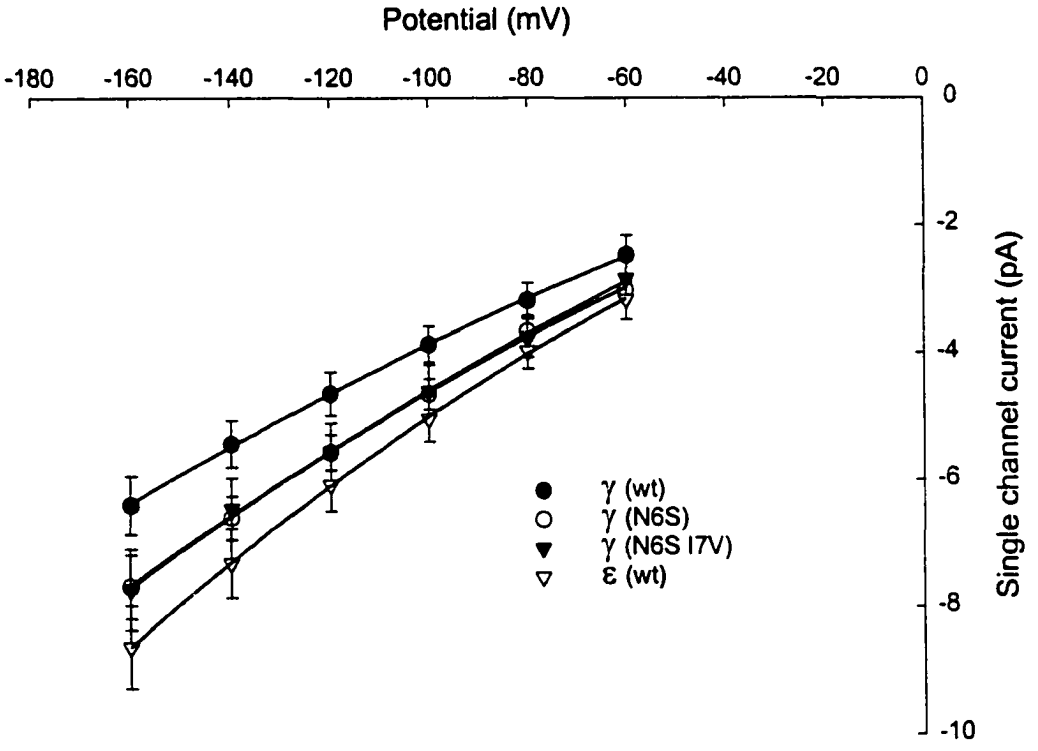

Figure 16 - Effect of γ M2 mutations on channel slope conductance.
Symbol legend indicates the constructs injected in combination with wild type α, β, and δ subunits. See Figure 15 legend for curve fitting procedures. Note overlap of curves for γ(N6S) and γ(N6S I7V). The estimated slope conductances were: γ (wt) 37.3 pS; γ (N6S), 45.2 pS; γ (N6S I7V), 46.2 pS; ε (wt), 51.9 pS.

Effects of M2 flanking domain mutations on channel conductance

We next set out to examine the proposed effects of the flanking regions on channel conductance. In addition to the original experiments comparing γ(wt) to γ(k21m) as described above, we also performed experiments comparing a complete swap of M2 region and flanking domains between ε and γ. We found that the addition of γ 18, 21 and 22 residues to the ε(s6n v7i) mutant resulted in a further reduction of slope conductance to the level of γ(wt)(i.e. 38pS). Performing the converse experiment did not yield the expected opposite result. When γ(n6s i7v) was mutated to include a complete ε M2 region (i.e. γ(εM2f)), the resulting slope conductance was not as great as that obtained with just the γ(n6s i7v) construct. The γ(εM2f) mutant revealed a slope conductance of 42 pS versus the 46 pS channel for the γ(n6s i7v) alone.

CHAPTER 4
DISCUSSION

The data presented in this thesis provide plausible explanations of the changes in acetylcholine receptor channel conductance and open time, which take place during the development of amphibian skeletal muscle. The developmental shift in channel conductance and open time can almost completely be attributed to two amino acid residue substitutions that occur during development. The replacement of asparagine at position 6 in the embryonic γ subunit with serine in the adult ε subunit accounts for the entire open time difference and 60% of the conductance difference between the receptors. Conversely, replacement of asparagine for serine at position 6, and valine for isoleucine at position 7 in the adult ε receptor, reverses all of the developmental change in open time and 75% of the conductance change which normally occurs during development.

Conductance

Having determined some of the important structural elements responsible for the developmental changes in the acetylcholine receptor, we examined alternative hypotheses which might explain how these few residues could account for the observed changes. With regard to conductance, imaging studies of the *Torpedo* acetylcholine receptor in its open state (Unwin, 1995) led to the hypothesis that fixed

charges within the pore, attractive to cations, as well as steric hindrance of ion movement, were responsible for differences in channel conductance. Unwin's experiments showed that when the channel opens, residues at positions 2, 6 and 10 of each subunit turn towards the center of the channel pore. This results in the narrowing of the open channel at the cytoplasmic side. The narrowest constriction of the channel is thought to be formed by the threonine residues at position 2 (Villarroel *et al.*, 1991; Unwin, 1995). In this model, the residues at position 6 would be near the narrowest part of the pore and would be in a position to interact with cations passing through the channel.

Figure 18 illustrates the molecular modeling of the M2 domain of the ε(wt), (s6n) and (s6n, v7i) mutants (Swiss Pdb Viewer, Glaxo Inc). The model predicts that the amide oxygen of asparagine will project about 0.1nm further into the channel pore than the hydroxyl oxygen of serine. This could result in an increased steric hindrance to ion movement through the channel. The increased hindrance to flow could explain the lower conductance of the asparagine containing embryonic γ channel.

The charge difference between the asparagine and serine residues may also contribute to the conductance differences. The carbonyl oxygen atom of the asparagine amide group bears about 0.5 negative charge (Abeles *et al.*, 1992). The hydroxyl oxygen of serine bears a predicted charge of 0.2 (Zumdahl, 1993). We predict that the greater negative charge of the asparagine residue would result in stronger hydrogen bonding

to the hydration sphere of cations traversing the channel. This stronger bonding would likely increase the period of time in which the cations are contained within the channel. The end result would be an increase in transit time and therefore a decrease in conductance in the γ(wt) embryonic channel.

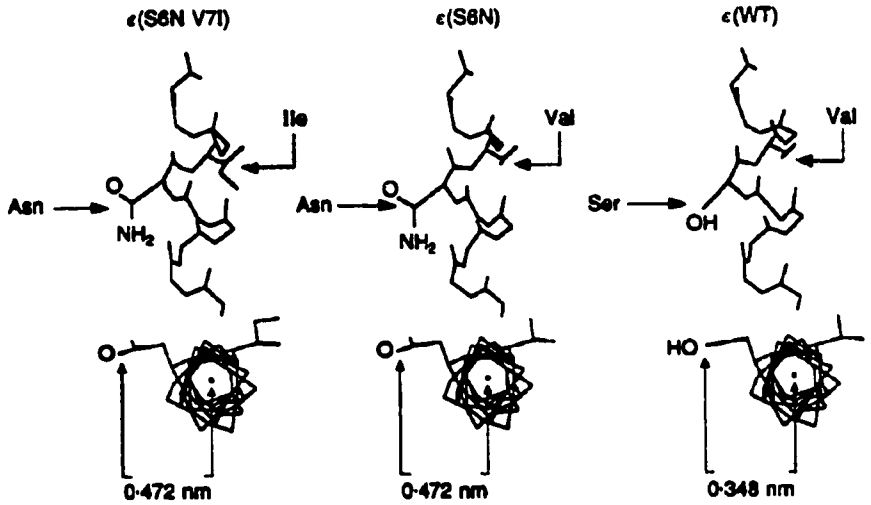

Figure 17 - Longitudinal and axial views of α-helical M2 domain.
Note that the asparagine residue projects 0.472 nm into the lumen of the channel, as compared to 0.348 nm for serine. Swiss Pdb Viewer (Glaxo Inc.) was used to model the helix.

Open Time

The structural determinants of channel open time cannot be explained as simply as those governing conductance. We found that the same structural elements at position 6 in the M2 region exerted effects on both conductance and open time, but we also found that other positions influenced open time. The M2 region, the M2 flanking regions, the HA region and PKA phosphorylation sites were all shown to be determinants of channel open time.

Studies in mammals have shown channel open time to be multi-factorial as well. Of interest is a study in humans in which an M2 region mutation was responsible for prolonged channel open time in Slow-Channel Syndrome (Milone *et al.*, 1997). In this study, an M2 mutation that did not face the channel lumen resulted in a prolonged open time. Other studies have implicated the M4 region and the M3-M4 linker regions as important areas for modulating open time. (Bouzat *et al.*, 1998; Bouzat *et al.*, 2000; Grosman *et al.*, 2000).

One possible explanation for the observation that multiple residues influence channel open time lies in the thermodynamics of transitions between the open and closed state of the receptor channel. All of the mutations described above will alter the conformation of the channel protein to some degree. These conformational changes could influence open time by increasing the activation energy barrier between open

and closed states. Increasing the activation energy barrier would decrease the rate at which the channel moves from an open to a closed state, thereby increasing the dwell time of the channel in an open state.

Another possible but less likely explanation for the finding that multiple regions elicit changes in open time lies in the binding affinity of the acetylcholine neurotransmitter to the receptor. If the open time of the channel is determined by the length of time that the acetylcholine neurotransmitter molecule is bound to the receptor, then changing receptor affinity to acetylcholine would directly affect channel open time. As discussed in the background, the neurotransmitter binds in a cleft region between the α and δ subunit on one side of the channel, and between the α and theγ or ε subunit on the opposite side. If channel open time is determined by agonist/receptor affinity, then one possible explanation for the developmental change in open time is that the conformational change that occurs when switching from a γ to an ε subunit results in weaker binding of the acetylcholine neurotransmitter to the subunit complexes. This possibility is suggested by mammalian studies showing a different acetylcholine binding affinity for different subunit complexes (Sine, 1998). If the alpha-gamma binding site had a stronger affinity for acetylcholine than the alpha-epsilon complex, the acetylcholine molecule would remain bound longer, resulting in a prolonged open time. Conversely, the weaker alpha-epsilon composition would result in a more rapid release of the neurotransmitter and result in more rapid closure of the channel. If this is true, it would be remarkable that a single residue substitution within the channel

pore, far-removed from the acetylcholine binding site, could affect acetylcholine binding affinity. The conformational changes in the HA region, M2-M3 linker region and M4 region could also exert their effects on open time via similar conformational mechanisms, that is, by modulating the affinity of the receptor for acetylcholine.

In addition to structural mechanisms, our data suggest that different phosphorylation sites and degrees of phosphorylation of the γ and ε receptor channels may also play a role in controlling channel open time. We found that phosphorylating agents had small but significant effects on channel open time. There was considerable variability in the effects of phosphorylating agents, which could be due to variable phosphorylation of PKA or PKC sites on other subunits. In addition, we did not experimentally confirm that phosphorylation occurred in response to treatment with phosphorylation agents. However, the observed effects, although small and variable, support the idea that post-translational events, such as phosphorylation, may modulate channel open time.

Finally, in discussing the present data, there are inherent errors of our mutagenesis experiments that must be taken into consideration. Uncertainties in interpreting our data could result from the fact that mutant residues in the M2 region interact with other wild type residues in the same subunit, most likely with those in the other membrane spanning domains. Such interactions could have unpredictable effects on the geometry of the pore. We could be in error when we assume, for example, that a

gamma (n6s i7v) construct has a tertiary conformation in the region of position 6 and 7 which is identical to that of a wild type epsilon subunit. In fact, because the mutant residues are exposed to other gamma wild type residues, rather than those of epsilon, the resulting conformation of the gamma M2 may not resemble either wild type gamma or wild type epsilon. Unexpected interactions between mutant and wild type residues within the gamma subunit could explain our difficulties in interpreting the conductance experiments, where mutation of residues at positions 6,7,18, 21 and 22 in the γ-subunit did not produce as much increase in conductance as mutation of residues at positions 6 and 7 alone. These five mutations produce a stretch of the γ-subunit that has the same sequence as the ε-subunit but not necessarily the same geometry. This inherent problem with mutagenesis experiments might underlie the fact that Herlitze *et al.* (1996) found that they had to replace all four membrane-spanning segments to completely switch conductance properties between γ- and ε-subunits.

Conclusion

The purpose of this thesis was to identify the structural elements underlying the observed functional differences between embryonic and adult amphibian acetylcholine receptor channels. We showed that single residue substitutions at crucial locations in the channel pore resulted in significant changes in channel conductance and open time. We also proposed molecular mechanisms to explain these functional changes. These results offer new molecular insights into the classic observation that synaptic currents become briefer in duration during the course of muscle development in vertebrates.

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